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(57) Abstract

The present invention relates to phospholipids with a desired carboxylic acid residue, such as an ω-3-fatty acid residue, in the 2-position. These compounds are produced by esterifying a conventional lysophospholipid with the corresponding carboxylic acid in the presence of the catalyst phospholipase A2, the esterification taking place in a microemulsion with a water content not exceeding 0.1-2 % by weight.

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A METHOD FOR THE PREPARATION OF A PHOSPHOLIPIDE WITH A CARBOXYLIC ACID RESIDUE IN THE 2-POSITION AND A PHOSPHOLIPIDE WITH AN ω-3-FATTY ACID RESIDUE IN THE 2-POSITION

The present invention relates to a method for the preparation of a phospholipid with a selected carboxylic acid residue in the 2-position by esterification in a microemulsion with the aid of a specific enzyme, phospholipase A2. The invention also concerns a phospholipid with an ω-3-fatty acid residue in the 2-position.

Many hydrolytic enzymes have both high activity and high stability in microemulsions, i.e. thermodynamically stable solutions of a hydrophobic component, water, and a surface-active component. In studies focussing on lipase, it has been found that a high water content in the system promotes hydrolysis, whereas a low water content, preferably below 1% by weight, results in the opposite reaction, i.e condensation. Lipase-catalysed esterification of glycerol and fatty acid in a microemulsion with low water content is described in literature (Fletcher et al., Biochim. Biophys. Acta 912(1987)278; Bello et al., Biochem. Biophys. Res. Comm., 146(1987)361).

As to triglycerides, it has been found that a small addition of a triglyceride with a different fatty acid composition, e.g. containing an unusually short, an unusually long, a branched or an unusually unsaturated fatty acid, markedly affects such physico-chemical properties as crystallisation and softening ranges, as well as lubricating and friction-reducing qualities. Furthermore, it would seem that the nutritional properties are much altered already by minor changes in the fatty acid composition of the triglycerides.

Much less study has been devoted to phospholipids, mainly because it is difficult to prepare substances with a specific fatty acid composition. However, it is very likely that small variations in the fatty acid composition

would lead to the same type of qualitative changes as for the triglycerides.

Recently, medical interest has focussed on the ω-3-fatty acids which is the generic term for polyunsaturated fatty acids which have 18-22 carbon atoms and whose last double bond, as counted from the carboxyl group, is between the third and the fourth carbon atom as counted from the methyl group end of the fatty acid molecule. A connection has been shown between a high intake of ω-3-fatty acids and a reduced frequency of heart and vascular diseases. An augmented intake of ω-3-fatty acids reduces the cholesterol content of the blood, and ω-3-fatty acids are therefore often prescribed for people with blood counts indicating an increased risk of thrombosis and infarct of the heart. The ω-3-fatty acids are normally available not only in the form of triglycerides from e.g. cod-liver oil, but also in the form of free fatty acids usually extracted from fish oils. In the human body, the triglycerides are metabolised, and part of the fatty acids are incorporated in the cell membranes of the body, a main component of these membranes being phospholipids. However, this incorporation is a slow process and only a minor amount of the added ω-3-fatty acids is incorporated in the membranes, regardless of whether they originally had the form of triglycerides or free fatty acids. Therefore, there is a great need for products which contain ω-3-fatty acids and can be taken up by the body in a more efficient manner.

With the aid of a specific enzyme, phospholipase A2, it has now proved to be feasible to esterify the 2-position of a lysophospholipid by adding a carboxylic acid. Normally, phospholipase A2 hydrolyses the ester bond of the phospholipid in the 2-position, but under the conditions prevalent during the inventive esterfication, the enzyme esterifies a lysophospholipid in the 2-position, surprisingly enough. This reaction takes place in a micro-emulsion. Since the phospholipid is surface-active in

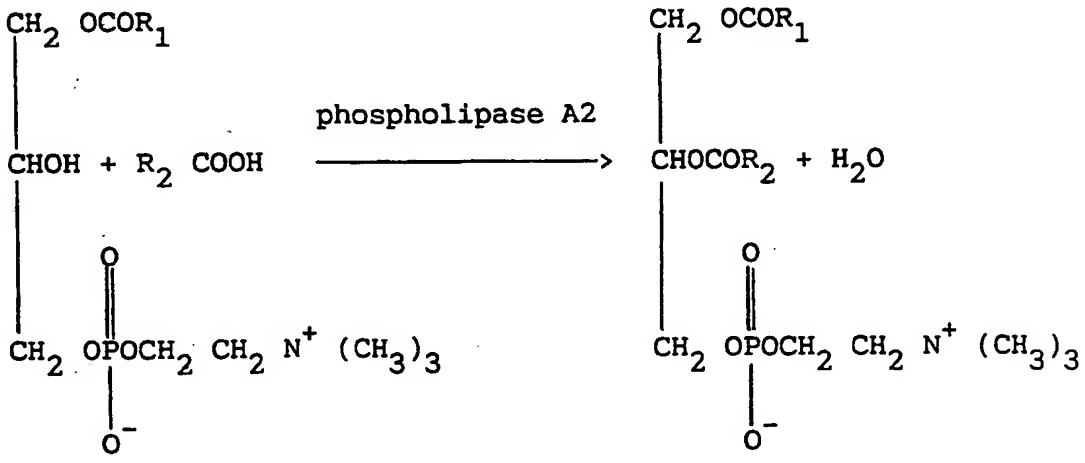
itself, it should theoretically be able to form by itself a microemulsion together with the hydrophobic component and the water. In practice, however, it is preferred that the lysophospholipid is supplemented with at least one other surface-active compound, preferably an anionic tenside. Preferably, the hydrophobic component consists of aliphatic hydrocarbons, but it may also be a supercritical solvent, e.g. carbon dioxide. The water content is low, usually 0.1-2% by weight, preferably 0.5-2% by weight.

The added carboxylic acid, which preferably is aliphatic, may be straight or branched, saturated or unsaturated. The number of carbon atoms of the fatty acid may vary within wide limits, but the range of 10-22 carbon atoms has attracted the greatest interest. With the invention, it is evidently possible to prepare a very broad spectrum of phospholipids. Suitable carboxylic acids include decanoic acid, dodecanoic acid, tetradecanoic acid, hexadecanoic acid, octadecanoic acid, oleic acid, ricinoleic acid, linoleic acid, linolenic acid, abietic acid, and dehydroabietic acid. The ω-3-fatty acids, which above all are to be found in algae and fish oils, are especially preferred. The most common ω-3-fatty acids, i.e. eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are ideal as reactants in the inventive reaction.

From the medical point of view, the phospholipids with ω-3-fatty acid residues in the 2-position obtained according to the invention are extremely advantageous compared with conventional fatty acid and triglyceride products. Since phopholipids are a biologically active part of the cell membranes of the cellular body, no metabolism need take place in the body, which should increase the efficiency of the ω-3-fatty acid. It is to be understood that the invention does not require pure products. Usually, the lysophospholipids are mixtures of several different components, and most of them have a structure which resembles that of the diglycerides but in which a fatty acid group has been replaced by an organic

group derived from phosphoric acid and a nitrogen base. Phosphatidyl choline is usually a main component. Furthermore, there are varying amounts of several closely-related substances, such as lysophosphatidyl ethanolamine, lysophosphatidyl serine, and lysophosphatidyl inositol. Generally, the added ω-3-fatty acid is not pure, but consists of a mixture of different fatty acids, such as EPA and DHA, and further contains a fairly significant amount of fatty acids other than the ω-3-type. Even if pure ω-3-fatty acid were to be used in the inventive reaction, the incorporation in the phospholipid would not be complete, since the esterification is an equilibrium reaction. All in all, the ω-3-fatty acid-containing phospholipid referred to in this context may consist of a large number of different substances. However, a distinctive feature is that a fairly significant proportion of the phospholipid, i.e. at least 10% and usually 15% or more, contains ω-3-fatty acid in the 2-position.

If desired, the inventive reaction may be illustrated by the reaction formula of lysophosphatidyl choline.



wherein R_1 is an acyclic hydrocarbon residue which contains 11-21 carbon atoms and is not being of ω-3-type, and R_2 is a polyunsaturated ω-3-fatty alkyl group with 17-21 carbon atoms.

The esterification according to the present invention is normally carried out at a temperature of between 20°C and 60°C. The aliphatic hydrocarbons, such as isooctane and nonane, forming the hydrophobic component, constitute 65-98% by weight of the composition. Supercritical solvents, such as carbon dioxide, can be used as hydrophobic component instead of the aliphatic hydrocarbons, in which case the reaction takes place at an elevated pressure. Usually, the water is buffered to pH 6-10. In addition to the lysophospholipid, both ionic and nonionic tensides may serve as surface-active component. Suitable ionic tensides include sodium dioctyl sulphosuccinate and fatty acid soaps, and suitable nonionic tensides include monoglycerides, sucrose fatty acid esters, sorbitan esters, and ethoxylated sorbitan esters. Frequently, mixtures of different tensides are to be preferred when formulating microemulsions. Suitably, the amount of lysophospholipid and ω-3-fatty acid added makes up 1-20% of the total composition. The surface-active components used should meet food standard requirements. Furthermore, a suitable content is 0.1-10% by weight of the total composition.

In a special embodiment, a conventional phospholipid first undergoes enzymatic hydrolysis to become a lysophospholipid, whereupon esterification as above is carried out. The net result of the two reaction steps is a transesterification, i.e. a replacement of an ordinary fatty acid residue in the 2-position by another carboxylic acid residue, e.g. an ω-3-fatty acid residue. The same enzyme, phospholipase A2, serves as catalyst in both the hydrolysis step and the condensation step, and microemulsions are a suitable medium for both processes. To guide the course of the reactions in the desired direction, the first step, the hydrolysis reaction, is suitably carried out in a microemulsion with a higher water content, e.g. 2-5%, than the second step, the esterification reaction, which may take place in a medium with a water content of 0.5-2%. If desired, the two reaction steps may be combined

to form a single process in which phospholipid and carboxylic acid together with an enzyme are added to a microemulsion with the higher water content and in which the water content is gradually reduced by stripping under vacuum, or by adding a hydrophilic substance, e.g. zeolite. Even if the water content is not varied, a certain amount of phospholipid containing the desired carboxylic acid residue in the 2-position can be obtained, but the yield is usually poor and the reaction time long.

Preferably, the reaction is made to take place in a proctective atmosphere and in the presence of an antioxidant in order to avoid autoxidation of the polyunsaturated fatty acids. Suitable antioxidants include tocopherol, butyl hydroxyanisole, butyl hydroxytoluene, and ascorbic acid. Combinations of at least one lipophilic and at least one hydrophilic antioxidant have at times proved advantageous.

The invention will be illustrated in more detail by the following Examples.

Example 1

The following composition was used:

Component	% by weight
Isooctane	87.3
Sodium dioctyl sulphosuccinate	3.4
Lysophosphatidyl choline	4.0
ω-3-fatty acid	4.0
Aqueous buffer, pH 8.2	1.3

To the above composition which, at 30°C, was a limpid isotropic solution, was added phospholipase A2 in an amount of $2.5 \cdot 10^4$ units/g lysophospholipid. The reaction was allowed to continue at 30°C under N_2, the solution being continuously stirred. After 16 h, the reaction was interrupted. The phospholipid was isolated by chromatography on a silica column, and the fatty acids were set free by hydrolysis and methylated, whereupon the esters were analysed by gas chromatography. The 10-metre silica columns used in the gas chromatography had an inner dia-

meter of 0.32 mm, Carbowax 1.2 µm serving as a stationary phase. Nitrogen gas under a pressure of 5 psi and a flow rate of 120 ml/min. was used as carrier gas. The column temperature was 220°C, and the injector temperature was 275°C. By means of the gas chromatogram, it was determined that more than 90% by weight of the phosphlipid contained ω-3-fatty acid residues. The reaction of lysophospholipid gave a 10% yield of phospholipid.

Example 2

The same composition as in Example 1 was used, except that the tenside was made up of a mixture of sorbitan monostearate and polyoxyethylene (20) sorbitan monostearate, the molar ratio being 1:2.

After a 24-hour reaction during which the same amount of enzyme was added and the same conditions prevailed as in Example 1, a phospholipid fraction, of which more than 90% contained ω-3-fatty acid residues, was obtained in a 7% yield.

Example 3

The same composition as in Example 1 was used, except that isooctane was replaced by n-heptane, and a phosphate buffer of pH 7.0 was substituted for the phosphate buffer of pH 8.5.

After a 3-hour reaction during which the same amount of enzyme was added and the same conditions prevailed as in Example 1, a phospholipid, of which more than 90% contained ω-3-fatty acid residues, was obtained in an 8% yield.

Example 4

The following composition was used:

Component	% by weight
Isooctane	87.0
Sodium dioctyl sulphosuccinate	7.0
Phosphatidyl choline	3.0
Aqueous buffer, pH 7.0	4.0

To this composition, $CaCl_2$ was added to a concentration of 10 mM and phospholipase A2 in an amount of $1.5 \cdot 10^4$ units/g phosphatidyl choline. The reaction was allowed to continue for 16 h at 30°C, whereupon the water content was reduced to 1.5% by weight by an addition of zeolite. ω-3-fatty acid in an amount corresponding to 6% by weight of the composition was added. After a further 16 h at 30°C, the reaction mixture was processed as in Example 1. The amount of phospholipid containing ω-3-fatty acid residues was found to be 58% by weight.

Example 5

The following composition was used:

Component	% by weight
Isooctane	87.3
Sodium dioctyl sulphosuccinate	3.4
Lysophosphatidyl choline	5.0
Dodecanoic acid	3.0
Aqueous buffer, pH 8.2	1.3

To the above composition which, at 30°C, was a limpid isotropic solution, was added phospholipase A2 in an amount of $2.5 \cdot 10^4$ units/g lysophospholipid. The reaction was allowed to continue at 30°C under N_2, the composition being continuously stirred. After 16 h, the reaction was interrupted. The phospholipid was isolated by chromatography on a silica column, and the fatty acids were set free by hydrolysis and methylated, whereupon the esters were analysed by gas chromatography. The 10-metre silica columns used in the gas chromatography had an inner diameter of 0.32 mm, Carbowax 1.2 µm serving as a stationary phase. Nitrogen gas under a pressure of 5 psi and a flow rate of 120 ml/min. was used as carrier gas. The column temperature was 275°C. With the aid of the gas chromatogram, the amount of phospholipid containing a dodecyl group was determined to more than 90% by weight. The reaction of lysophospholipid gave a 12% yield of phospholipid.

Example 6

The same composition as in Example 5 was used, except that dodecanoic acid was replaced by γ-linolenic acid, and the contents of fatty acid and lysophosphatidyl choline were altered from 3.0% and 5.0%, respectively, to 4.0% for both.

After a 16-hour reaction during which the same amount of enzyme was added and the same conditions prevailed as in Example 5, a phospholipid fraction, of which more than 90% contained γ-linolenic acid residues, was obtained in a yield of 11%.

Example 7

The same composition as in Example 5 was used, except that 11-methyl dodecanoic acid was substituted for dodecanoic acid.

After a 6-hour reaction during which the same amount of enzyme was added and the same conditions prevailed as in Example 5, a phospholipid, of which more than 90% contained 11-methyl dodecanoic acid, was obtained in a yield of 7%.

CLAIMS

1. A method for the preparation of a phospholipid with a carboxylic acid residue in the 2-position, c h a r a c t e r i s e d in that a lysophospholipid is esterified with a corresponding carboxylic acid in the presence of the catalyst phospholipase A2, the esterification taking place in a microemulsion with a water content of 0.1-2% by weight.

2. Method as claimed in claim 1, c h a r a c t e r i s e d in that the carboxylic acid is an aliphatic carboxylic acid with 10-22 carbon atoms.

3. Method as claimed in claim 1 or 2, c h a r a c t e r i s e d in that the carboxylic acid is an ω-3-fatty acid.

4. Method as claimed in any one of claims 1-3, c h a r a c t e r i s e d in that the surface-active component of the microemulsion comprises, apart from the lysophospholipid, at least one nonionic or anionic surface-active compound, or mixtures thereof, in an amount of 0.1-10% by weight of the total composition, and that the hydrophobic component of the microemulsion constitutes 65-98% by weight of the total composition.

5. Method as claimed in claim 3 or 4, c h a r a c t e r i s e d in that the lysophospholipid and the ω-3-fatty acid are added in an amount of 1-20% by weight of the total composition.

6. Method as claimed in any one of claims 3-5, c h a r a c t e r i s e d in that the ω-3-fatty acid contains 18-22 carbon atoms.

7. Method as claimed in any one of claims 1-6, c h a r a c t e r i s e d in that the lysophospholipid is largely made up of lysophosphatidyl choline, lysophosphatidyl ethanolamine, lysophosphatidyl serine and lysophosphatidyl inositol, or mixtures thereof.

8. Method as claimed in any one of claims 1-7, c h a r a c t e r i s e d in that the lysophospholipid is obtained by an enzymatic hydrolysis of the corresponding phospholipid in a microemulsion with a water content of 2-3% by weight.

9. A phospholipid c h a r a c t e r i s e d by an ω-3-fatty acid residue in the 2-position.

10. Phospholipid as claimed in claim 9, c h a - r a c t e r i s e d in that the ω-3-fatty acid has 18-22 carbon atoms.

11. Phospholipid as claimed in claim 9 or 10, c h a r a c t e r i s e d in that it is largely made up of phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, or phosphatidyl inositol.

12. Phospholipid as claimed in any one of claims 9-11, c h a r a c t e r i s e d by a concentration of at least 10% by weight.

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE 90/00481

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 P 7/64, C 11 C 3/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 11 C; C 12 N; C 12 P

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

SE,DK,FI,NO classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	Chemical Abstracts, Vol. 90 (1979), abstract No 50071c, R. W. Evans et al. Chem. Phys. Lipids 1978, 22(3), 207-20 (Eng.) --	9
Y	Chemical Abstracts, Vol. 73 (1970), abstract No 84125n, H. P. Franck et al., Z. Naturforsch B 1970, 25(6), 581-6 (Ger.) --	1-8
Y	Chemical Abstracts, Vol 69 (1968), abstract No 168k, H. P. Franck et al. Z. Naturforsch. B 1968, 23(4). 43948 (Ger.) --	1-8
Y	Chemical Abstracts, Vol. 82 (1975), abstract No 53462b, Ronald L. Misiorowski et al., Biochemistry 1974, 13(24), 4921-7 (Eng.) --	1-8

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
12th October 1990	1990 -10- 15
International Searching Authority	**Signature of Authorized Officer**
SWEDISH PATENT OFFICE	Yvonne Siösteen

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A2, 0265699 (THE NISSHIN OIL MILLS) 4 May 1988, see the whole document --	9
A	SE, B, 452166 (BEROL KEMI AB) 16 November 1987, see the whole document --	1-8
A	FR, A1, 2599382 (INSTITUT DES CORPS GRAS -ITERG.) 4 December 1987, see the whole document --	1-8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.PCT/SE 90/00481

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on 90-08-28
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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SE-B- 452166	87-11-16	EP-A-B- 0237092	87-09-16
		SE-A- 8601222	87-09-11
		US-A- 4839287	89-06-13
FR-A1- 2599382	87-12-04	NONE	